EXHIBIT B

Targeting of B cells in SLE

Rationale and Therapeutic Opportunities

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Abstract

B cells were first implicated in lupus pathogenesis because of their roles as autoantibody producers. B cells, which play important roles in (auto)immune recognition, are now understood to also have other functional capabilities that contribute to the recruitment and stimulation of T lymphocytes and cells of the innate immune system. Herein, these emerging insights are discussed as well as the newly recognized influence on B cells of the Toll-like receptors, which are postulated to be important sources of costimulation for autoreactive B cells. Also discussed is how B-cell survival factors may contribute to the loss of immune tolerance that leads to pathologic autoimmunity. These findings are part of the rationale for the development of new specific B-cell-targeted therapies.

Systemic lupus erythematosus (SLE) represents a systemic autoimmune disease that can affect many organ systems, and its pathogenesis is known to involve many cell types of the innate and adaptive immune systems. Autoantibodies and immune complexes have long been known to be centrally involved. However, in recent years our perspectives on the pathogenesis of the disease have matured. In the following sections I discuss how classical perspectives on autoimmunity have evolved to encompass several other potential functional roles of B-lineage cells believed to be involved. These insights have provided the rationale for the development of new therapeutic B-cell-targeted approaches for the treatment of patients with lupus.

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Classical Views of B Lymphocytes as Sources of Autoantibodies

Earlier investigations of renal pathogenesis in SLE primarily focused on the roles of autoantibodies in the development of immune complex-mediated glomerulonephritis. Affected patients were shown to produce autoantibodies with a wide array of specificities. While many types of autoantibodies have not been clearly proven to be directly involved in pathogenesis, a very large literature shows that IgG anti-native-DNA antibodies can have pathogenetic potential for the development of proliferative lupus nephritis (reviewed by Diamond et al[1]). Exploiting this relationship, tests for anti-native-DNA antibodies have become widely accepted as adjuncts for the diagnosis of SLE in the rheumatology clinic. Patients with high-titer anti-native-DNA autoantibodies generally have a worse prognosis and often require more aggressive immunosuppressive therapy than those without such antibodies.[2] Even so, many SLE patients with anti-native-DNA antibodies do not develop lupus nephritis, which suggests that all DNA-reactive antibodies may not necessarily have equivalent pathogenetic potential and/or that other factors also contribute to renal pathology.

To investigate the molecular basis for DNA recognition by lupus autoantibodies, antibody genes from a large number of human and murine anti-DNA antibodies from hybridomas have been characterized. Based on the prominence of high somatic hypermutation in these antibody gene sequences, it was earlier postulated that anti-DNA lupus B-cell clones are the products of germinal center (GC) responses in peripheral lymphoid tissues suggesting that SLE is associated with a defect in peripheral immune tolerance. However, it remains possible that such B-cell responses bypass GC checkpoints, and may at times not be dependent on T-cell help. In any case, a recent report

of single cell analysis of circulating B cells from pediatric SLE patients has provided further evidence of defects in peripheral deletion of autoreactive clones.[3]

Certain recurring patterns of antibody gene somatic mutations have been shown to correlate with enhanced DNA-binding capacity. However our recent pilot studies have shown that the same mutations that enhance binding to native-DNA also convey broad cross-reactivity with a range of other self-proteins (manuscript in preparation, G. Silverman). These findings are consistent with reports that many nephritogenic anti-native-DNA antibodies also bind to non-DNA determinants prevalent in glomeruli.[4,5] In pursuit of a better test for nephritogenic autoantibodies, Gilkeson and coworkers have developed an assay that detects IgG specific for determinants in DNAse-treated glomerular extracts. This assay may provide advantages in assessing disease susceptibility and perhaps also relative activity of lupus nephritis.[6,7] In support of this approach, we have performed surveys of human lupus sera with a multiplex autoantigen microarray system. Using this technique, we confirmed that the presence of renal disease correlated well with high antibody reactivity towards a glomerular extract.[8]

Emerging Perspectives on Immune Complex-Mediated Pathogenesis

While some controversy remains as to how autoantibodies induce glomerular disease, one hypothesis postulates that autoantibodies form immune complexes at remote sites, which are subsequently deposited in the kidney. However, as suggested above, the consensus view now favors that such complexes are formed in situ in the kidneys when circulating autoantibodies form immune complexes with cognate antigens that are expressed in the kidney (i.e., presumably exposed and accessible in the glomerulus). Antibodies trigger effector functions via specific sites in their heavy chain constant regions. When immune complexes are formed, some immunoglobulin isotypes can fix complement by the classical pathway, and the induction of an unimpeded cascade of local complement activation leads to the production of chemotactic factors, such as C5a, which attract the leukocytes that further hasten downstream tissue destructive activities. In addition, complement fragments associated with immune complexes also activate cells such as macrophages and dendritic cells through specific complement receptors (e.g., Mac-1, complement receptor 3, CR3 and CD11c, CR4).

The special proinflammatory potential of many IgG autoantibody immune complexes also derives from their capacity to trigger immune cells through membrane-associated Fcγ receptors (reviewed in Ravetch and Boland[9]). Complement and FcγR pathways have been shown to be highly interconnected, as the release of the downstream active complement factor, C5a, can enhance expression of Fcγ receptors responsible for IgG-immune complex cellular triggering.[10] Hence, in a variety of ways, autoantibodies play central roles in the self-perpetuating mechanisms underlying pathologic autoimmune response.

Despite all that we know about autoantibodies, the specificities and levels of circulating IgG autoantibodies are not the only determinants of autoimmune disease activity. In a recent small open-label trial, the treatment of active lupus nephritis patients with the chimeric anti-TNF antibody, infliximab, greatly decreased laboratory markers of inflammation and also improved renal function, despite the fact that levels of anti-native-DNA and anticardiolipin antibodies were unaffected or even increased.[11] This report supports the paradigm that the in vivo pathogenetic potential of autoantibodies is largely dependent on local proinflammatory conditions and/or that immune complex-mediated tissue injury may be mediated by the induction of TNFα.

Beyond Autoantibodies: Other Functional Roles for B Cells

Lund and coworkers used an in vitro murine model system to show that activated B cells are capable of producing a wide range of cytokines.[12,13] Analogous to helper effector T cells that express distinct cytokine profiles, mature B cells may also have the potential to differentiate into parallel subsets of B cells that produce either Th1-biased (e.g., IFNγ and TNFα) cytokines or Th2-biased (e.g., IL-4, IL-10, and IL-6) cytokines. Moreover, B-cell production of IL-6 and IL-10 may provide both autocrine and paracrine functions that further sustain B-cell-mediated immune responses. Therefore, an interdependent costimulation of parallel sets of recruited B cells and T cells may commonly contribute to (auto)immune responses. Speculatively, such polarized B cells may be important for the initial recruitment of T cells, and the reciprocal functional contributions of these B cells and T cells could be central to the self-perpetuating nature of chronic autoimmune disease processes.

First described more than 20 years ago, B cells can serve as very efficient antigen-presenting cells (APC), which likely are also important for autoimmune pathogenesis.[14,15] In contrast to professional APC (e.g., macrophages and dendritic cells) that take up local antigens through their cellular membranes by nonspecific transport mechanisms, B cells specifically trap antigen through their membrane-associated antigen receptors (i.e., surface immunoglobulin or B-cell receptor [BCR]). This antigen encounter also cross-links the membrane-associated BCR complexes, which induces both B-cell activation and internalization of the BCR-antigen complexes for processing and presentation of derived antigenic peptides. Significantly, because the BCR binds antigen with micromolar to sub nanomolar affinities, a B cell can take up, process, and present an antigen with

an overall efficiency that is 1000- to 10,000-fold greater than for a professional APC, so that even autoantigens at very low local concentrations may be efficiently presented to recruited specific T cells. Highly relevant to many autoimmune diseases, B cells that express BCR with anti-IgG specificity (i.e., rheumatoid factors [RF]) represent greater than 5% of healthy repertoires, and this may be further increased in certain autoimmune diseases (reviewed in Chen[16]). Because RF-bearing B cells can take up, process, and present any (auto)antigen in an IgG-immune complex, they may be prime contributors to chronic autoimmune processes by their efficient autoantigen presentation and costimulation of autoreactive T cells.

Toll-like Receptors and B-cell Survival Factors in Lupus Pathogenesis

In SLE and other autoimmune diseases, IgG-associated immune complexes may contain nonprotein self-components that themselves also provide immunomodulatory capacities. For more than 20 years, it has been known that lupus patients often have high circulating levels of type I interferon (IFN).[17,18] However, the underlying mechanisms remained undefined until it was suggested that type I IFN could be induced by the nucleic acid-containing IgG immune complexes found to be common in the circulation of SLE patients.[19] Significantly, a class of ancient receptors of the innate immune system, termed Toll-like receptors (TLR), recognize both microbial and, in some cases, self-ligands, including some forms of RNA and DNA (reviewed in Kawai et al[20]).

Triggering via TLR delivers stimulatory signals that result in enhanced leukocyte activation and secretion of cytokines, including type I and II interferons. Hence, B cells that bear autoreactive BCR (i.e., RF, anti-DNA or anti-RNA, and others) may take up autoantigens from the breakdown products from dying cells. These affected B cells and neighboring affected cells are induced to express membrane-associated cognate molecules, like B7 family (e.g., CD86, CD80), ICOS-L, CD40, and other molecules that are important for providing the second signal to stimulate T cells. The TLR of B cells may be stimulated by these nuclear components to produce costimulatory signals, which contribute to breaches in immune tolerance and perpetuate the chronic proinflammatory phase of disease.[21] These events can result in chemokine production, recruitment of macrophages, and ultimately, in the case of kidney disease, glomerular damage.

IgG-nucleic acid complexes, in one form or another, may also stimulate a specialized form of dendritic cell, termed a plasmacytoid dendritic cell, that is believed to be the most important source of IFN.[22,23] Type I IFN drives the maturation of conventional myeloid dendritic cells as well as drives B cells to differentiate to plasma cells. Dendritic cell stimulation also leads to the release of chemokines and cytokines, such as the B-cell pro-survival factors BAFF (B-cell activating factor, also known as BLyS, TALL-1, THANK, and zTNF4) and the related TNF member, APRIL,[24] which play important and specific roles in the survival of autoreactive B cells.[25] Hence, several inflammation-associated pathways lead to the release of factors that enhance the survival of autoreactive B cells, resulting in impaired immune tolerance.

Infiltrative B Cells and Ectopic Lymphoid Aggregates

B cells also constitutively express a cytokine, termed lymphotoxin (LT) $\alpha_1\beta_2$, that can engage LTβ receptor on stromal cells and cells of myeloid lineage and induce the secretion of a chemoattractant implicated in the development and organization of peripheral lymphoid structures. Moreover, LT has also been implicated in the development of the ectopic lymphoid infiltrates that arise in chronic infectious diseases like hepatitis C. These factors are believed to contribute to the development of ectopic lymphoid infiltrates commonly seen in the affected synovium of patients with rheumatoid arthritis (RA), but may also occur in other autoimmune diseases, such as neo-lymphogenesis in the salivary glands in Sjögren's patients and in thyroiditis patients. While much less is currently known about such processes in lupus, the kidneys of lupus-prone NZB/NZW mice have been shown to fill with plasma cells that are major sources of autoantibodies.[26]

While related literature in clinical lupus is currently limited,[27,28] available studies indicate that the kidneys of lupus nephritis patients often have tubulointerstitial lymphocyte infiltrates, although their histologic organization is often loose; the highly refined architectural features of GCs may be relatively uncommon.[29] How such local B-cell infiltrative processes may contribute to the clonal selection and expansion of autoreactive B cells remains to be defined. Speculatively, these B cells later may enter draining lymph nodes where, as sources of autoantibodies, they contribute to lupus pathogenesis. They may also contribute to autoimmune pathogenesis through other cell-mediated activities as described above.

Concluding Remarks

Autoimmune pathogenesis in SLE involves complex intercellular-dependent pathogenetic processes, and B lymphocytes appear to play central roles by contributing several coordinated functional capabilities, all within one cellular package. These insights have provided the rationale for the recent development of agents that specifically target B cells for depletion in patients with SLE and other autoimmune diseases. Current and future agents target B cells either via specific cell membrane-associated determinants (Fig. 1) or by blockade of B-cell

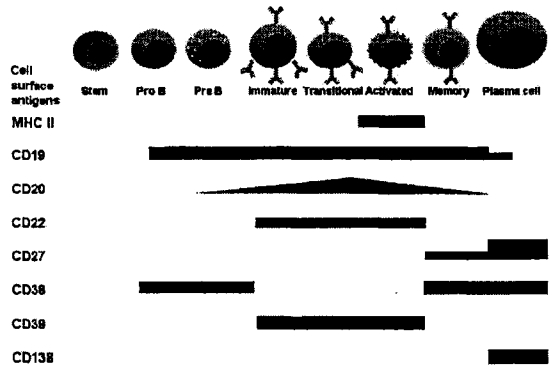

Figure 1 Distinct stages of B-cell maturation as identified by differential expression of patterns of surface phenotype markers. In adults, B-cell progenitors and precursors are produced in the bone marrow, leading to the differentiation of transitional B cells that are released into the bloodstream before they enter peripheral lymphoid organs. In patients with rheumatoid arthritis, transitional B cells, aberrant related B-cell precursors, recirculating follicular B cells, and plasma cells may enter inflamed joints in response to chemokine gradients along high endothelial venules. MHC II indicates class II major histocompatibility complex. (Reprinted with permission from Silverman.[41])

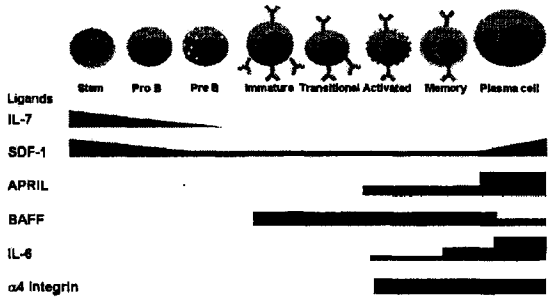

Figure 2 Variation in responsiveness to survival factors during B-lineage cell maturation and differentiation. While most observations have been made in murine models, human B cells may differ from mouse B cells, and certain subtleties may currently be undefined. For example, although interleukin-7 (IL-7) is essential for B-cell maturation in mice, it is not essential in humans, although human pre-B cells express IL-7 receptors and respond to IL-7 in the presence of stromal cells. The role of 4 integrin in human B-cell survival remains to be confirmed. While IL-6 is required for in vitro survival of murine plasma cell, IL-6–deficient mice have normal Ig levels, but different outcomes could be induced with therapeutic blockade. General principles of the patterns of responsiveness to pro-survival factors are represented, but the details for certain factors may not be well defined. SDF-1 indicates stromal cell–derived factor 1. (Reprinted with permission from Silverman.[41])

survival factors (Fig. 2).

Of the several potential strategies for the targeting of B cells, targeting via their BCR may provide special advantages, as this strategy theoretically would be able to deplete B cells that express only certain binding (auto)specificities or only certain variable region elements, without affecting other B cells that are required for immune defenses. Selective BCR targeting is believed to be involved in the mechanism of action of the synthetic oligomeric DNA agent abetimus (LJP394), which was developed to deplete peripheral anti-DNA B cells in SLE patients.[30]

In our laboratory, we have investigated the mechanism(s) of action of the bacterial toxin staphylococcal protein A (SpA) that targets many B cells for death. We have shown that SpA induces the apoptotic death of only B cells that express targeted V_H3-containing B-cell antigen receptor (BCR) by a mitochondrial signaling pathway that induces cell death[31,32] (reviewed in Silverman and Goodyear[33]). Significantly, SpA is also the active moiety in an apheresis column that is approved by the Food and Drug Administration (FDA) for the treatment of the autoimmune diseases, RA and thrombocytopenia.[34] While it has been shown that hundreds of micrograms of SpA leach off the column and are infused into the patient during each treatment with this device, the immunobiologic implications were unsuspected until we demonstrated that infusions of even microgram doses of this protein can induce quantitative in vivo B-cell depletion[35] (discussed in Silverman et al[36]). Moreover, SpA infusions have been shown to ameliorate murine SLE.[37] We have therefore proposed that SpA, or therapeutic analogues that target other B-cell subsets, might provide therapeutic benefits for SLE patients.

As an alternate strategy to targeting B cells, therapeutic agents are being developed to interfere with critical B-cell-specific survival factors. Phase II studies in SLE have recently been completed with a blocking antibody, termed belimumab, to BAFF. This antibody demonstrated a high level of safety and some efficacy.[38] Additional antibody-based and decoy-receptor agents against BAFF and APRIL are also in development.

The greatest progress in B-cell depletion therapy has been made in studies of rituximab, a chimeric antibody to the B-lineage-specific surface molecule CD20. In the United States, rituximab was first FDA approved in 1997 for the treatment of non-Hodgkin's lymphoma,[39] and based on results from a blinded, controlled phase III trial, was approved in early 2006 for the treatment of RA patients with inadequate response to TNF blockers.[40]

By selectively perturbing only the B-cell compartment of the immune system, these therapeutic agents may also help us to better understand autoimmune pathogenesis. While rituximab almost uniformly induces blood B-cell depletion in RA patients, the responses are more heterogeneous in SLE patients; much remains to be learned

about the immunologic implications of these treatments (as discussed in Silverman[41]). Initial phase I/II results with rituximab in SLE patients are encouraging.[42-45] While the safety profile appears attractive, clinical progress has been slowed by difficulties in the design of SLE trials and in defining treatment regimens. However, these areas are advancing rapidly, and based on interim progress (as recently discussed in Silverman[46]), there is much hope that safer and more effective therapies that target B cells will soon become available for the treatment of SLE.

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